# VIRULENCE AND TOXIN-FORMATION IN B. DIPHTHERIAE[*]

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## INTRODUCTION

The virulence of B. diphtheriae and organisms closely related to it morphologically and culturally, is of great importance in the application of quarantine laws. This is especially emphasized by the increasing evidence that many apparently healthy individuals harbor in the nose and throat diphtheria bacilli which are capable of causing an acute attack of diphtheria in nonimmune individuals. Kolmer and Moshage[1] state that 5-20% of healthy individuals harbor the bacilli in the throat, and 10-20% carry them in the nose. In 30-80% of such individuals giving no history of diphtheria, the cultures are avirulent. In individuals who have come into intimate contact with diphtheria cases or have had the disease themselves, the bacilli are virulent in 61-100% of cases. It has been realized for many years that the ability of certain strains of B. diphtheriae to produce potent toxins on suitable fluid media does not always run parallel with the virulence of such organisms. This observation has been made especially in connection with Park's No. 8 strain of the diphtheria bacillus, which is in common use in antitoxin laboratories and which produces a potent toxin. This particular strain was isolated about 20 years ago by Dr. Park from a rather mild clinical case of diphtheria. This fact alone would not be sufficient basis for ascribing a low grade of virulence to the strain; for virulence is not dependent on one factor, but on many. Since, however, the strain has retained its strongly toxigenic properties on artificial media for 20 years and at the same time has shown slight virulence for the guinea-pig, it is evident that the toxin-producing powers of this strain cannot be taken as indicative of virulence. The work presented in this paper was undertaken to discover, if possible, some of the relations between toxin-formation and virulence.

Virulence as usually conceived is the disease-producing power of an organism, and is dependent in general on 2 factors—the organism's

ability to multiply and produce a toxin and its invasive power.   The
virulence of an organism, then, may be modified by changing its power
to produce toxin in the animal host or by any treatment that may influ-
ence its power of invasion or of multiplication.   Numerous methods
have been devised for the accomplishment of this purpose.

The passage of bacteria through a susceptible host has been shown by many
investigators to increase its virulence for that host.   It does not always fol-
low that its virulence is increased simultaneously for another host.   Thus
Ohlmacher[2] passed a nonvirulent diphtheria strain through a guinea-pig and
recovered a virulent diphtheria strain, and Salter[3] passing the pseudodiphtheria
bacillus through canary birds, thereby increased its virulence.   Clark[4] and others
have been unable to confirm these results by identical methods.   It has been
established without question, however, that M. pneumoniae increases in viru-
lence on passage through mice.   Staphylococci from boils or carbuncles are
recognized as being more virulent than are staphylococci which have lived as
saprophytes for a long time.   The explanation often advanced for this increased
virulence is that of prolonged natural selection; that is, in a large number of
organisms introduced there are some weak ones which are killed off by the
protective mechanism of the invaded host, leaving only the more resistant to
survive.   By repeated passage a race is finally developed which may possess many
times the virulence of the original culture.   That animal passage may some-
times have the opposite effect is shown by the passage of smallpox virus
through young calves, which reduces its virulence not only for human beings,
but for calves as well.

It is interesting to note the possible relation of capsule-formation to viru-
lence.   Certain microorganisms, notably M. pneumoniae, B. anthracis, and some
streptococci, show an increasing tendency to capsule-formation following ani-
mal passage and a simultaneously increasing virulence.   This is apparently
a protective measure on the part of the bacteria against the resistant agencies
of the host.   It has been pointed out by several workers[5] that the slimy cap-
sulated organisms are not amenable to agglutination, and that they are taken
up by the leukocytes less readily than uncapsulated organisms.   Even when
a distinct capsule is not visible, according to Eisenberg,[6] the more virulent
organisms may show an ectoplasmic hypertrophy comparable to capsule-
formation.

The path of entry and the number of organisms introduced undoubtedly
play an important part in the establishment of a disease by microorganisms.
The path through which the bacteria enter and the place where they find
lodgment must furnish suitable environmental and cultural conditions for their
existence and multiplication if the organisms are to manifest their virulence.
In diseases in which the bacteria invade the blood stream and give rise to a
general bacteriemia (such as typhoid fever, anthrax, and plague), the bacterial
virulence is undoubtedly due in part to this ability of the specific organism to
penetrate into the blood stream.   In other diseases, such as diphtheria and

[2] Jour. Med. Research, 1902, 2, p. 128.

[3] Tr. Jenner Inst. Prev. Med., 1899, 1 (2nd series), p. 113.

[4] Jour. Infect. Dis., 1910, 7, p. 335.

[5] Shibayama, Centralbl. f. Bakteriol., I, O., 1905, 38, p. 482.   Porges, Wien. klin.
Wchnschr., 1905, 18, p. 691.   Gruber and Futaki, München. med. Wchnschr., 1906, 53,
p. 249.

[6] Centralbl. f. Bakteriol., 1908, 45, p. 638.

tetanus, in which the bacterial growth is localized and in which bacteriemia rarely has been shown, the virulence of the organism must be due to a different set of causes. Even when introduced through the most favorable channels, an organism usually virulent may fail to show any pathogenic powers. This may be due to a number of influences including individual resistance to the microorganism and other immune factors. But it may also be due to the quantity of organisms that is introduced. Experiments carried out by Webb, Williams, and Barber[7] indicate that the number of anthrax bacilli necessary to produce a fatal infection depends on the virulence of the strain. They found that 3-6 anthrax bacilli taken directly from the blood of a dead animal produced fatal results, while 10-25 times as many taken from an old agar culture were harmless. Also, in working with B. tuberculosis, they found that tuberculosis could be produced with 20 bacilli of one strain, while 150 were required of another. The initial dosage, in order to cause infection, must be sufficiently large to overcome any slight natural resistance on the part of the host. It is evident that this amount varies inversely with the virulence.

It has been stated by Bail[8] that some varieties of highly virulent bacteria produce substances, which he terms 'aggressins,' that are responsible for the high degree of virulence possessed. He believes that the germicidal activity of the body fluids in natural immunity has been over-emphasized, and that certain characteristics possessed by different strains of bacteria which render them insusceptible to phagocytosis play an important rôle in the infectivity of the organism. He has shown that the peritoneal fluid from guinea-pigs dying after a fatal injection of typhoid or cholera organisms possesses the power to increase the infectivity of homogeneous strains thàt would otherwise be harmless. Thus, when otherwise sublethal doses are introduced together with such peritoneal fluid, the organisms produce a fatal infection. Even tho the animal at the same time be injected with a protective bacteriolytic serum, death may yet be produced when the bacteria and peritoneal exudate are introduced together. In short he holds that the bacteria produce protective bodies for themselves, enabling them to multiply in the host by combating its defenses. These antibodies he believes are in the nature of antiopsonins which protect the bacteria from phagocytosis. By injecting the aggressin exudate into animals, Bail was able to produce an antiaggressin which rendered the bacteria defenseless and permitted phagocytosis. Many[9] believe that these aggressins are nothing more than endotoxins which have a negative chemotactic influence and not a specific action. The 'virulins' of Rosenow[10] are apparently very similar in nature to Bail's 'aggressins.' Rosenow found that freshly isolated cultures of pneumococci were not phagocytable, but that this property was lost on subsequent subculture. By extraction with salt solution he obtained a substance from virulent strains which he termed 'virulin,' that increased the virulence of avirulent pneumococci by rendering them less phagocytable. Whatever may be thought regarding the nature of these substances, it is evident that there are bacterial products formed by some varieties of bacteria which do influence their virulence.

Closely related to this production of defensive agents by bacteria is the

[7] Jour. Med. Research, 1909, 20, p. 1.

[8] Arch. f. Hyg., 1905, 52, p. 272.

[9] Wassermann and Citron, Deutsch. med. Wchnschr., 1905, 31, p. 28. Kolle and Wassermann, Deutsch. med. Wchnschr., 1905, 31, p. 1101. Doerr, Centralbl. f. Bakteriol., I. O., 1906, 41, pp. 497, 593.

[10] Jour. Infect. Dis., 1907, 4, p. 285.

hypothesis of Welch.[11] Welch believes that when bacteria are grown in their own immune sera, they develop more resistance to these sera by some mechanism and become more virulent. Experimental evidence in corroboration of this theory has been produced by Walker.[12] By cultivating B. typhosus in its immune serum, he destroyed its power of agglutination (made it immune) and increased its virulence. The immune serum in which the bacilli were grown became at the same time less agglutinative and protective against the bacilli.

The diphtheria group of bacilli have been classified (1) on a morphologic basis, (2) on the basis of biometric reactions, and (3) on the basis of results in virulence tests on guinea-pigs. It is recognized that the morphologic appearance of the organism cannot be relied on entirely for classification within the group, even tho it is of fundamental importance.

### THE BIOMETRIC REACTIONS OF B. DIPHTHERIAE

The biometric reactions of the group have been carefully studied by several workers[13] with a view to differentiation of B. diphtheriae, B. hofmanni, B. xerosis, and other diphtheroids. All are agreed that true diphtheria bacilli produce acid in dextrose media and usually from glycerin, and that B. hofmanni produces little or none. With other sugars a less constant reaction has been observed.

Knapp,[13] Zinsser,[14] Morse,[13] and others found that in saccharose broth acid was not produced. Martin,[15] however, found that acid was produced, while Kolmer and Moshage[13] found that a small percentage of cultures from the nose and throat fermented saccharose. The formation of acid with the latter sugar is considered important in differentiating B. xerosis from other members of the group. Martin found that in maltose broth acid was not formed but Morse reports a production of acid. Dextrin, according to Morse, is fermented by some strains and not by others, while according to Martin acid is produced from this sugar by all true diphtheria bacilli. In general the conclusions seem to indicate that the monosaccharids are fermentable by true diphtheria bacilli, but that the higher sugars are fermented less characteristically and less uniformly.

The biometric experiments here recorded were made for the purpose of determining the possible relation between virulent and non-virulent types. It was expected that all would ferment dextrose and glycerin, but that there might be a difference in the degree of acidity or in the fermentation of other sugars that would be of differential value.

[11] Brit. Med. Jour., 1902, 2, p. 1105.
[12] Jour. Pathol. and Bacteriol., 1902, 8, p. 34.
[13] Knapp, Jour. Med. Research, 1904, 12, p. 475. Lubenau, Arch. f. Hyg., 1908, 66, pp. 305, 335. Morse, Jour. Infect. Dis., 1912, 11, p. 253. Heine, Jour. Pathol. and Bacteriol., 1913-14, 18, p. 75. Kolmer and Moshage, Jour. Infect. Dis., 1916, 19, p. 1.
[14] Jour. Med. Research, 1907, 17, p. 277.
[15] Ann. de l'Inst. Pasteur, 1898, 12, p. 26.

Little previous work has been done to determine this point. Moshage and Kolmer[13] state that virulent diphtheria bacilli are more prone to ferment carbohydrates than nonvirulent, and they suggest that acid-production may run parallel to toxin-production. Goodman[16] found by continued selection of high- and low-acid transfers that from the same original strain low- and high-acid strains could be obtained, and that the virulence for guinea-pigs was much greater in the high-acid strains than in the low. Heine[17] attempted to distinguish virulent from avirulent bacilli by dividing the morphologically true types of bacilli into 3 groups according to their biometric reactions. The first group, or true virulent diphtheria bacilli, ferments dextrose, maltose, lactose, and dextrin, but not saccharose; the second group, often isolated from the skin, ferments dextrose, saccharose, and sometimes maltose, but not dextrin or lactose; and a third group, often found in the urogenital tract, ferments dextrose, maltose, dextrin, and saccharose, but not lactose.

In order to get the optimal conditions for acid-production with a minimal amount of labor, some preliminary studies were made regarding the influence of oxygen tension, of the incubation period, and of certain carbohydrates. They are incorporated in this paper, even tho they do not bear directly on the main theme.

*Materials for Study and Methods.*—All but 4 of the 26 cultures used in this work were freshly isolated from serum smears taken in clinical cases of diphtheria. The four strains were taken from old stock cultures. In isolating from mixed cultures, it was sometimes possible to transfer from a single isolated colony, but in most instances it was found necessary to transfer some of the mixed culture to fresh Loeffler's blood serum, a small amount of the culture being spread over 3 successive slants without reinoculation of the needle. This was done by side sweeps of the needle from the bottom to the top of the tube. Usually individual colonies of diphtheria bacilli could be fished after 24 hours' incubation and, after determining their purity by strains, transferred to sterile Loeffler's slants. Three colonies could be studied on the same cover slip by employing the technic of Graham Smith.[18] This consists in placing 3 small loops of water on a cover slip, inoculating each from a different colony of bacteria, streaking in parallel lines, drying, fixing, and mounting in Loeffler's methylene blue diluted 1:5. This method has given very satisfactory, rapid results. When a colony was found containing a pure culture of B. diphtheriae, a transfer was made from it to Loeffler's blood serum. After 24 and 48 hours' incubation the culture was studied in methylene blue stain for the types of bacteria present (Wesbrook's classification). It was then transferred to 1% glycerin agar, litmus milk, and gelatin for further confirmation before being tested biometrically on 1% dextrose, maltose, saccharose, dextrin, and glycerin broths.

*Relation of Oxygen to Acid-Production.*—The first series of experiments was carried on in small 60-c.c. Erlenmeyer flasks, 1% dextrose broth with an initial reaction of + 0.5 being used. To one series of flasks 5 c.c. of broth were added and to the other series 30 c.c., making a depth of 3-5 mm. and an exposed surface of 17.5 sq. cm. in the first instance, and a depth of 2 cm.

16 Jour. Infect. Dis., 1908, 5, p. 421.
17 Jour. Pathol. and Bacteriol., 1913-14, 18, p. 75.
18 Nuttall and Graham Smith, The Bacteriology of Diphtheria, 1908.

with an exposed surface of 14.4 sq. cm. in the latter. These were sterilized in the Arnold sterilizer and inoculated at the same time from vigorously growing broth cultures of Strain 0 and Park's Strain 8, and incubated at 37 C. with uninoculated controls. Titrations were made in duplicate after 6, 12, 24, 48, and 72 hours. An average of the two titrations was taken (unless they varied widely, whereupon other tests were made) and the titer of the control flask, obtained at the same time, was deducted from it. All titrations were made with phenolphthalein as an indicator, the culture having been boiled for 1 minute in a casserole. Previous to titration, the organisms were killed by heating in the Arnold sterilizer for 20 minutes. At the outset it was questionable whether titrations should be made without heating or according to standard methods. It was realized that there was a possibility of losing volatile acids by heating. A number of tests, made by titrating some in the cold and some after boiling for 1 minute, showed very slight differences. This same result was obtained by Moshage and Kolmer,[19] who state that little difference in acidity is to be observed between cultures tested before and those tested after boiling for 2 minutes.

The results, given in Table 1, seem to indicate that a large exposure to the air increases the rate of production of acid. This is especially marked during the first 24 hours, but is still true at 72 hours.

TABLE 1

The Relation of Oxygen to Acid-Production by B. Diphtheriae *

| Time (hr.) | Strain 0 | | Park's Strain 8 | |
|---|---|---|---|---|
| | 5 c.c. | 30 c.c. | 5 c.c. | 30 c.c. |
| 6 | 0.1 | 0.0 | | |
| 12 | 0.6 | 0.0 | 2.0 | 1.2 |
| 24 | 2.5 | 1.0 | 4.8 | 3.6 |
| 48 | 4.2 | 2.6 | 4.8 | 4.0 |
| 72 | 4.7 | 3.8 | | |

* 1% dextrose broth used. Results in percentages normal acid.

Further tests bearing on the same point were made by employing Nessler tubes, 17 mm. in diameter, containing 10, 20, 30, and 40 c.c. of dextrose broth. These amounts made a depth of broth in the respective tubes of 4.5 cm., 9 cm., 13.5 cm., and 18 cm. respectively, which gave varying conditions of aerobiosis. Two strains were employed for this experiment, Strain 1 and Park's No. 8. With both strains 1% dextrose broth was used with an initial reaction of — 0.5 with the first strain, and + 0.5 for the other strain. The medium was prepared and titrated with the sugar added and then sterilized in the Arnold sterilizer. The tubes were inoculated from 24-hour vigorously growing broth cultures and incubated at 37 C. Two tubes with controls were titrated on alternate days, after killing the organisms by boiling the culture for 5 minutes on the water bath. The titrations throughout were made as indicated, and the results obtained by averaging the titers of two tubes and deducting the reaction of the sterile control. The purpose in this experiment was to determine the time of maximal acidity in each group of tubes and its relation to the exposure to oxygen of the air. The results are shown in Table 2. Strain 1 showed a greater amount of acid the 2nd day in the tubes containing the smaller amounts of broth, or in those having relatively the greatest amount of exposure

[19] Jour. Infect. Dis., 1916, 19, p. 19.

to the air. The maximal acidity in the tubes containing 10 c.c. was reached on the 8th day and in the tubes containing 20, 30, and 40 c.c. on the 12th day. In Strain 8 the maximum was reached the 6th day in the 10-c.c. tubes, the 8th day in the 30-c.c. tubes, and the 10th day in the 20- and 40-c.c. tubes. While the results obtained on the relation of oxygen to acid-production are admittedly too meager on which to formulate any general principles, it is evident that the greater the exposure to oxygen the more rapid is the production of acid. It is also evident that for each strain there is a maximal acidity which is reached eventually in 1% dextrose broth regardless of the exposure to oxygen.

TABLE 2

TIME OF MAXIMAL ACIDITY IN 1% DEXTROSE BROTH WITH VARYING OXYGEN TENSION

| Amount (c.c.) | Days | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 |
| Strain 1. Initial Reaction — .5 | | | | | | |
| 10 | 2.0 | 2.45 | 2.95 | 3.6† | 2.9 | 3.35 |
| 20 | 1.9 | 2.65 | 2.7 | 3.15 | 2.9 | 3.7† |
| 30 | 1.75 | 2.75 | 3.2 | 3.1 | 3.05 | 3.4† |
| 40 | 1.15 | 2.45 | 2.1 | 3.1 | 2.9 | 3.2† |
| Strain 8. Initial Reaction +.5 | | | | | | |
| 10 | 2.3 | 4.4 | 5.0† | 4.6 | 4.6 | |
| 20 | 2.4 | 4.7 | 4.8 | 4.8 | 4.9† | |
| 30 | 3.1 | 4.6 | 4.1 | 5.0† | 5.0 | |
| 40 | 3.1 | 4.9 | 4.4 | 4.6 | 5.0† | |

Results in percentages normal acid.     † Maximal acidity.

*Time of Maximal Production of Acidity in Various Carbohydrates.*—It has been indicated by the work of Morse[20] that the maximal acidity produced by B. diphtheriae is reached in the different sugars at a rather constant time interval for the various carbohydrates. She found that the greatest acidity in dextrose occurred on the 13th day; in maltose and dextrin, on the 8th or 9th day; and in glycerin, on the 15th or 16th day. In view of the probable effect of aerobiosis on acid-production, it was deemed advisable to obtain further evidence under the conditions which were to be used in future work, relative to the time of maximal acid-production in the various carbohydrates. The experiments were conducted with 1% of the carbohydrates in infusion broth with an initial reaction of + 0.5, dextrose, maltose, saccharose, dextrin, and glycerin being employed. Ten cubic centimeters of the sugar broths were placed in tubes with a uniform bore of 16 mm., making a depth of about 5.5 cm. These were sterilized in the Arnold sterilizer, inoculated from a 24-hour broth culture, and incubated at 37 C. Two strains, Nos. 0 and 8, were used. Every day 2 tubes of each sugar from the two strains with controls were titrated, after boiling for 5 minutes to kill the organisms. This was continued daily for 15 days. It will be seen from Table 3 that the results with the two strains did not coincide in all respects. A slight acidity developed in the case of Strain 8 on saccharose broth the 1st day; otherwise no acidity was found, but a marked alkalinity was prevalent throughout. In dextrin a preliminary acidity

[20] Jour. Infect. Dis., 1912, 11, p. 253.

was found for 2 and 3 days respectively, followed by an alkaline reaction. This is in accord with Morse's observations. She found that saccharose was never fermented and dextrin only by some strains. In maltose some acid was formed early until the 6th or 8th day and then there followed an alkaline reaction. In dextrose a high acidity was produced rapidly; the reaction then fluctuated between high acidity and the maximum throughout the entire time, the maximum of Strain 0 being reached on the 9th day and of Strain 8 on the 4th day. Acid was formed in glycerin broth rather slowly by both strains and reached a maximum on the 14th day in each strain.

TABLE 3

TIME OF MAXIMAL ACIDITY IN 1% CARBOHYDRATE BROTHS, INITIAL REACTION +0.5

| Carbohydrate | Days | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| Strain 0 | | | | | | | | | | | | | | | | |
| Dextrose | 1.0 | 2.7 | 2.8 | 3.3 | 3.3 | 3.6 | 3.5 | 3.5 | 4.1† | 3.9 | 3.9 | ... | 4.0 | 4.0 | 4.1† | 3.8 |
| Maltose | 0.6 | 1.4† | 0.5 | 0.0 | 0.2 | 0.1 | 0.1 | 0.2 | 0.1 | 0.0 | 0.1 | ... | 0.3* | 0.1* | 0.4* | 0.8* |
| Saccharose | 0.2* | 0.2* | 0.5* | 0.2* | 0.6* | 1.1* | 1.2* | 1.6* | 1.5* | 2.3* | 2.1* | ... | ... | 2.2* | 1.9* | 2.6* |
| Dextrin | 0.1 | 0.6† | 0.1 | 0.3* | 0.3* | 0.6* | 1.3* | 0.9* | 1.4* | 1.8* | 1.6* | ... | ... | 1.8* | 1.8* | 2.0* |
| Glycerin | 0.1 | 0.1 | 0.3 | 0.2* | 0.3 | 0.4 | 1.6 | 1.8 | 1.8 | 1.8 | 3.4 | ... | 3.8 | 4.2† | 4.2 | 4.0 |
| Strain 8 | | | | | | | | | | | | | | | | |
| Dextrose | 0.9 | 1.6 | 5.4 | 6.3† | 6.3† | 6.0 | 6.0 | 5.7 | 5.5 | 5.5 | 5.9 | 6.5† | 5.9 | 6.2 | 5.7 | |
| Maltose | 0.3 | 1.0† | 1.0† | 0.5 | 0.3 | 0.3* | 0.4* | 0.4 | 0.1* | 1.6* | 1.6* | 0.9* | 1.5* | 1.2* | 1.2* | |
| Saccharose | 0.25† | 0.6* | 1.0* | 1.5* | 1.6* | 1.8* | 2.0* | 2.1* | 2.1* | 2.3* | 2.3* | 2.9* | 2.5* | 2.4* | 2.1* | |
| Dextrin | 0.2† | 0.0 | 0.3* | 0.5* | 0.5* | 1.1* | 1.0* | 1.4* | 1.8* | 1.3* | 1.9* | 1.8* | 1.8* | 1.7* | 1.6* | |
| Glycerin | 0.0 | ... | 0.3 | 0.9 | 1.9 | 1.6 | 2.6 | 2.3 | 2.7 | 3.9 | 4.4 | 4.6 | 4.4 | 5.2† | 4.0 | |

Results in percentages normal acid.       * Alkalinity.       † Maximal acidity.

From the results obtained it is evident that the time required for the maximal production of acid in the different sugars varies with different strains of diphtheria bacilli. It appears that by using a standard 8-day observation on all of the sugars, as accurate an index of their biometric power would be obtained as by using a different time limit for each sugar as Morse suggests. It also seems probable that some of the discrepancy between the results obtained by different workers on the fermentative action of B. diphtheriae on maltose and dextrin may be due to the period of incubation previous to reading of results. The use of litmus as an indicator in Hiss serum water medium would not record as delicate changes in reaction as does the use of phenolphthalein. The amount of carbohydrate added to the broth seems to make little difference, within limits, in the amount of acid formed. A series of tests showed that 0.5% of the various carbohydrates gave as high maximal acidity as 1%, except in the case of glycerin, in which the acidity seemed to develop more slowly.

In view of the preliminary work outlined, it seemed wise to employ, as standards for the biometric tests, tubes 16 mm. in diameter, each containing 10 c.c. of the broth, to which 0.5% of the carbohydrate had been added previous to sterilization in the Arnold sterilizer, and which showed an initial reaction of 0.5. The carbohydrates employed were

dextrose, maltose, saccharose, dextrin, and glycerin. These were inoculated from 24-hour broth cultures and incubated for 8 days before titrations. Titrations were made after boiling the cultures 1 minute; an average of 2 tubes was taken in each case and the titer of the control tube deducted from it for the final result.

In all, 26 strains were tested in this manner. The results are recorded in Table 4. From the summary in Table 5 it will be seen that

TABLE 4

BIOMETRIC RESULTS FOR 26 STRAINS OF MORPHOLOGICALLY TRUE DIPHTHERIA BACILLI

| Culture | Dextrose | Maltose | Saccharose | Dextrin | Glycerin |
|---|---|---|---|---|---|
| 0 | 3.7 | 1.5* | 1.6* | 1.7* | 2.4 |
| 122 | 3.9 | 1.4* | 1.4* | 0.9* | 1.7 |
| 905 | 3.7 | 0.9* | 1.0* | 0.2 | 1.8 |
| 668 | 4.0 | 0.6* | 0.7* | 1.2 | 1.1 |
| 461 | 3.7 | 1.2 | 0.3* | 0.0 | 2.0 |
| 1290 | 3.9 | 0.9 | 3.4 | 0.2* | 1.9 |
| 694 | 4.4 | 0.1 | 0.0 | 0.1* | 1.2 |
| 1308 | 4.3 | 0.8* | 1.2* | 0.4* | 3.5 |
| 1 | 2.5 | 1.2 | 0.0 | 0.7 | 0.5 |
| 1502 | 4.1 | 0.9* | 0.8* | 1.7* | 0.9 |
| 168 | 4.4 | 0.0 | 1.3* | 0.6 | 1.4 |
| 880 | 4.4 | 0.5 | 1.2* | 1.5* | 1.1 |
| 1853 | 4.5 | 1.1* | 1.9* | 1.7* | 0.4 |
| 15 | 3.3 | 1.2 | 0.5* | 0.0 | 0.5 |
| 540 | 4.4 | 1.4* | 1.6* | 1.5* | 1.1 |
| 42 | 4.6 | 3.3 | 4.0 | 1.6 | 3.7 |
| 600 | 4.0 | 0.9 | 1.3* | 0.6* | 0.5 |
| 1293 | 4.7 | 0.8* | 1.5* | 1.1* | 0.4 |
| 1441 | 4.6 | 0.0 | 1.7* | 1.5* | 1.3 |
| Park's  8 | 4.8 | 0.5 | 2.0* | 1.3* | 3.0 |
| 49† | 4.2 | 2.3 | 1.3* | 0.3 | 2.1 |
| 50† | 4.6 | 0.2 | 0.7* | 1.0 | 5.2 |
| 2059 | 3.75 | 1.1 | 0.1* | 0.6 | 0.9 |
| 11 | 4.0 | 3.4 | 4.0 | 0.0 | 1.9 |
| 13 | 3.7 | 0.75 | 0.9* | 0.2 | 0.8 |
| 20 | 3.65 | 0.7 | 0.5* | 1.5 | 1.05 |

Results in percentages normal acid.     * Alkalinity.
 † Furnished thru the kindness of Dr. Theobald Smith and described in "Notes on Two 'Atoxic' Strains of Diphtheria Bacilli" by Brown and Smith (Jour. Med. Research, 1914, 30, p. 443).

TABLE 5

SUMMARY OF BIOMETRIC RESULTS FOR 26 STRAINS

| Result | Dextrose | Maltose | Saccharose | Dextrin | Glycerin |
|---|---|---|---|---|---|
| Producing acid...... | 26 | 15 | 3 | 10 | 26 |
| Producing alkali.... | 0 | 9 | 21 | 13 | 0 |
| No change.......... | 0 | 2 | 2 | 3 | 0 |

all strains fermented dextrose — the low and high limits being 2.5% and 4.8% normal acid, respectively. Nineteen of the 26 fell between 3.5 and 4.5%. In maltose 15 formed acid, 2 produced no change, and 9 produced alkalinity. In saccharose 21 produced alkalinity, 2 remained unchanged, and 3 produced acid. It is interesting to note that in the case of those forming acid, a relatively large amount (over 3.4%) was

formed.  In dextrin 10 produced acid, 3 produced no change, and 13 produced alkalinity.  In glycerin all of the 26 strains produced acid. There was a wide range, however, in the amount of acid formed, from 0.4 to 5.2%.  Twenty produced 2% or below, while 6 produced over 2%.  From the results obtained with the 26 strains, morphologically true diphtheria bacilli, it is seen that all ferment dextrose and glycerin, the majority do not ferment saccharose, over half ferment maltose, and less than half, dextrin.

### VIRULENCE IN B. DIPHTHERIAE

The fact that in many cases after recovery from an attack of diphtheria, avirulent organisms are isolated, early led to the belief that through some unknown action of the body fluids the virulence of the organisms was lost.  More recently it has been shown that while 10-20% of well individuals harbor in the nose and throat avirulent organisms belonging to the diphtheria group, the larger percentage of typical diphtheria bacilli isolated after recovery from an attack of the disease are virulent.  In a study of 2774 children, Perkins, Miller, and Ruh[21] found that 105, or 4.07%, were carriers of virulent diphtheria bacilli, potentially capable of producing the disease.  As was stated in the early part of this paper, virulence may be due either to the ability of the organism to produce a toxin or to its invasive power or to the combined action of these two factors.  In some diseases which are characterized by a generalized toxemia, such as diphtheria and tetanus, the virulence of the organism may be largely dependent on its ability to produce a soluble toxin.  Since experiments have shown that in many strains of diphtheria bacilli the virulence, as shown by guinea-pig tests, gives little idea of the toxigenic powers of the strain, the reason for this virulence in relatively atoxic strains has been sought.  As yet no satisfactory answer has been given.  The diligent effort to modify the virulence of members of this group and to determine the relations existing between the various members, tho unsuccessful in these respects, has contributed much to our knowledge of virulence and of methods for its determination.  This problem has been attacked from several angles: (1) animal passage, (2) symbiosis, (3) prolonged cultivation on artificial media, (4) heating, (5) drying, and (6) growth with 'aggressins.'

*Methods of Conducting Virulence Tests.*—Guinea-pigs have been generally adopted for testing the virulence of diphtheria bacilli because these animals

[21] Jour. Infect. Dis., 1916, 18, p. 608.

seem to be susceptible in about the same degree as man. The methods that have been employed, however, have been various. As public health laboratories are often called on to make virulence tests for release from quarantine of carriers or convalescents, it is important that the tests employed be as delicate and rapid as possible.

Graham Smith[18] grew the pure cultures on sugar-free broth for 48 hours and injected guinea-pigs subcutaneously. Other workers[22] grew organisms on 1% dextrose broth with a reaction of — 0.5 for 48 hours, the tubes being slanted to give a maximal exposure to the air, and injected the unfiltered growth in amount equivalent to 0.5% of the body weight, subcutaneously into guinea-pigs weighing 250-300 gm. If at the end of 4-6 days the animal was alive, the culture was considered nonvirulent. These workers ascribed the virulence chiefly to the toxin produced. Arms and Wade[23] found by testing the virulence in a manner similar to that of Weston and Kolmer, that different colonies developing from the same case varied in their virulence, and they suggested that a number of colonies be tested before calling the organisms nonvirulent. Following clinical diphtheria the organisms recovered are usually virulent. This was early shown by Stone,[24] who found that in 14 of 18 cases giving positive cultures after 3 weeks the bacilli were still virulent, as proved when 1 c.c. of a 48-120-hour sugar-free-broth culture was injected subcutaneously. Similar results have been reported by many other investigators since that time. Morse[20] using guinea-pigs weighing 150-250 gm. and injecting 0.5% of their body weight of a 48-hour culture grown on sugar-free broth with a reaction of — 0.5, classed as virulent those strains that killed within 4 days with typical lesions. Zinsser[25] tested for virulence by intraperitoneal injections of the growth from a 24-hour agar slant washed down in 5 c.c. sterile broth. One-fourth cubic centimeter of such a suspension was injected for each 100 gm. of weight of the guinea-pig. The use of an emulsion from Loeffler's blood serum for virulence tests is practiced in many public health laboratories. If the virulence is due to the toxin formed in broth, as Weston and Kolmer have suggested, there may conceivably be wide differences in action and especially in rapidity of action, between living cultures washed from agar or serum and those from broth. More recently Kolmer and Moshage[1] made a comparative study of the various methods employed for the determination of virulence. Tests made with 0.1 c.c. of a 72-hour plain-dextrose-broth culture injected intracutaneously did not give as high a proportion of positive results as did the subcutaneous injection when 0.5% of the body weight of the animal was used, and the results were more uncertain to read. In the intracutaneous test, if positive, superficial necrosis was observed in 48-72 hours. The subcutaneous injection of 72-hour serum-broth cultures gave results as good as those from subcutaneous injection of washed down Loeffler media, but the latter accomplished the results in shorter time. The subcutaneous injection of 72-hour plain-dextrose-broth cultures proved superior to the intraperitoneal injection of 24-hour and 72-hour plain-dextrose-broth cultures. They concluded that when the time required and delicacy of action were considered, the subcutaneous injection of 0.4 c.c. of a 24-hour Loeffler slant growth was the most satisfactory. A modified Neisser technic was reported by Zingher and Soletsky[26] in making intracutaneous virulence tests. They washed down a 24-hour Loeffler tube with 25 c.c. of sterile salt solution and injected 0.1 of

[22] Weston and Kolmer, Jour, Infect. Dis., 1911, 8, p. 295.
[23] Jour. Am. Med. Assn., 1911, 56, p. 809.
[24] Jour. Med. Research, 1897-98, 2, p. 11.
[25] Ibid., 1907-08, 17, p. 277.
[26] Jour. Infect. Dis., 1915, 17, p. 454.

this intracutaneously.  The advantage of their technic was that 4-6 different cultures might be tested on the same guinea-pig.  A control animal was inoculated with the same amount of the suspension plus 0.5 c.c. of a 200-unit antitoxin serum.

*Methods of Modifying Virulence.*—Numerous attempts have been made in the past to modify the virulence of diphtheria bacilli.  Unfortunately in many of these experiments no attempt has been made to distinguish between the toxigenic power and virulence.  As has been shown in the case of diphtheria bacilli, there is an essential difference between organisms in this respect.  Very early in the development of knowledge concerning the diphtheria bacilli, Roux and Yersin[27] and Funck[28] observed that diphtheria ran a more severe course when streptococci were present in large numbers, and experiments convinced them that in some way association in growth with streptococci caused an increased virulence in B. diphtheriae.  Hilbert[29] found that this increased virulence was due to an increased ability to form toxin.  The increased toxigenic power Escherich[30] ascribed to passage through susceptible animals, while others[31] attributed the increase to the ability of the organism to proliferate more rapidly in a medium rendered more favorable by the growth of streptococci. Smirnow[32] states that in his work streptococci had an antagonistic effect on diphtheria bacilli on blood serum during 10-15 hours' growth, but that after 20-30 hours this antagonistic action was not evident and might favor the growth. Another worker[33] grew two morphologically typical, but avirulent, cultures through 90 generations with virulent streptococci, transplanting every 3-4 days, and induced no increase in virulence.

More effort has been put forth in the attempt to increase virulence by animal passage.  Trumpp[34] claims to have increased the virulence of an avirulent bacillus by passing it with toxin through guinea-pigs, to such a degree that a culture from the dead animal killed another guinea-pig.  Hewlett and Knight[35] claim to have converted a pseudodiphtheria bacillus into a diphtheria bacillus by animal passage and heating, while Salter[3] accomplished the same result by passage of the bacillus through canary birds.  By cultivating the bacilli in collodion sacks in the peritoneum of the rabbit, Martin[15] was able to increase the toxigenic power of diphtheria bacilli, including that of Park's No. 8, but not their virulence.  It is claimed also by Ohlmacher[2] that the virulence of an avirulent strain was increased by passage through 1 guinea-pig.  Unfortunately many of these results have not been confirmed, even tho carefully repeated by other investigators.  The attitude of a majority of workers is well summed up by Kolmer, Woody, and Moshage,[36] who state: "The great weight of experimental evidence is to the effect that diphtheria bacilli which have proved nonvirulent with every test, remain so indefinitely despite prolonged efforts to give them even feeble pathogenic powers." It is thought more probable by many that the virulence of slightly pathogenic strains may be modified.  It may well be that it is with such slightly pathogenic strains that

[27] Ann. de l'Inst. Pasteur, 1890, 4, p. 385.
[28] Ztschr. f. Hyg., u. Infektionskr., 1894, 17, p. 465.
[29] Ibid., 1898, 29, p. 159.
[30] Wien. med. Wchnschr., 1894, 11, p. 294.
[31] Gibier, Compt. rend. Soc. de biol., 1897, 4, p. 392.
[32] Jour. Med. Research, 1908, 18, p. 249
[33] Williams, Jour. Med. Research, 1902, 8, p. 83.
[34] Centralbl. f. Bakteriol., 1896, 20, p. 721.
[35] Tr. Brit. Inst. Prev. Med., 1897, 1 (1st series), p. 7.
[36] Am. Jour. Dis. Child., 1916, 11, p. 257.

early workers experimented. To quote the afore-mentioned authors again, "with these (strains with low pathogenicity) passage through animals or from throat to throat among men may readily enough restore a high degree of pathogenicity." In a long series of animal passages it may also be possible that another strain has been recovered at some stage, different from the one introduced, since members of this group are rather widely distributed.

A decreased virulence has been brought about by several methods. Artificial cultivation has apparently produced no lessened virulence in the case of Park's bacillus nor modified its toxigenic powers. It is stated in one case[37] that in 2 years' cultivation on artificial media the virulence was partially lost. Hewlett and Knight[35] report the reduction of virulence by heating for 17 hours at 45 C., but they were unable to repeat their results. In attempting to verify these findings, Williams[33] found no reduced virulence in diphtheria bacilli grown at 40-45 C., for several months. Roux and Yersin[27] found a slightly decreased virulence in dried membrane, while Abel[38] observed a similar quality in bacilli dried on silk threads for 86 days. A few instances of peculiar manifestations of low-grade virulence by diphtheria bacilli have been recorded. Abscesses were noticed by Cobbett[39] at the site of inoculation after the injection of nonvirulent organisms. From these abscesses he isolated diphtheria bacilli in purity. He reports having seen similar abscesses in guinea-pigs treated with large doses of virulent bacilli together with antitoxin and in an immunized horse treated with living bacilli. Councilman[40] reports an abscess around a hair follicle from which were isolated diphtheria bacilli that killed a guinea-pig in 48 hours. In a fatal case following typhoid fever a lung abscess contained B. diphtheriae. Heine[17] more recently has suggested that his second group may possess pyogenic properties. One test[41] to determine whether 'aggressins' such as Bail found were present in virulent diphtheria bacilli, gave negative results, as follows: Three guinea-pigs were injected with virulent diphtheria bacilli to obtain the aggressin. After death the peritoneal fluid was sterilized by filtration and injected with 2 avirulent strains. None of the animals was affected either with or without the aggressin.

While diphtheria is primarily a toxemic disease, a number of cases are on record in which an invasion of the blood stream has been observed. There has been little or no attempt to determine the relation of such penetration to the virulence of the organism. Mallory[42] states: "In many fatal cases the bacilli get into the blood and can be obtained in cultures from various organs. The order of relative frequency is as follows: liver, kidneys, spleen, heart's blood and very rarely the brain. The highest percentage of septicemia recorded is fifty, but in this series general septicemia was found only in about 20%."[42] Another worker[18] twice isolated virulent diphtheria bacilli during life from the cerebrospinal fluid of a child suffering from general miliary tuberculosis and tuberculous meningitis. There were no clinical signs of diphtheria, but the organisms were present in the throat. One investigator[43] isolated diphtheria bacilli from the blood during life in 1 of 18 cases. In this case there was also a streptococcal infection, which he thinks may have predisposed to invasion. The bacteria were found only at one time, 9 days before death. Another

37 Bardach, Ann. de l'Inst. Pasteur, 1895, 9, p. 40.
38 Deutsch. med. Wchnschr., 1895, 17, p. 545.
39 Jour. Hyg., 1901, 1, p. 497.
40 Jour. Med. Research, 1896-97, 1, p. 14.
41 Zinsser, Jour. Med. Research, 1907-08, 17, p. 277.
42 Quoted by Nuttall and G. Smith, The Bacteriology of Diphtheria, 1908.
43 Leede, Ztschr. f. Hyg. u. Infektionskr., 1911, 69, p. 225.

worker[44] found diphtheria bacilli 3 times in 187 patients. Postmortem examination having given negative results, he concludes that bacteriemia, when it does occur, is temporary. Morgan[45] reports a case in which diphtheria bacilli found in the blood proved nonvirulent when tested on a guinea-pig. It is believed by some[46] that there is a temporary bacteriemia associated with every case of diphtheria at some time. One case in support of this theory is described by Wade,[47] a diphtheria bacteriemia in which 1600 bacilli to the cubic centimeter of blood were found to be highly virulent and to produce a toxin fatal in 0.015-c.c. amounts for guinea-pigs. In a number of cases reported, neither the virulence nor toxicity of the isolated culture had been tested, so that the results are without meaning in correlating the virulence with these factors.

In this study the methods adopted for determining the virulence and penetration of the 23 cultures tested were as follows: Sugar-free infusion broth with a reaction of +0.5 was inoculated with the pure culture and incubated at 37 C. for 48 hours. Unless a vigorous growth took place, several transfers were made in the broth until a good growth was obtained. Of this living unfiltered culture 0.2 c.c. was employed for the initial dose. After the total volume had been brought up to 4 c.c. with sterile salt solution, it was injected subcutaneously by means of a Hitchen syringe into guinea-pigs weighing 250-300 gm. If the animal died within 4 days, no further injections were made; if death did not occur in this time, 4 c.c. of a similar broth suspension were injected in the same manner into another guinea-pig. When death occurred, the animal was examined, and unless the typical postmortem findings for diphtheria were present, the death was not regarded as one due to diphtheria bacilli. The findings were typical of toxemia in all reported cases, consisting of marked induration and hyperemia at the site of injection, abundant gelatinous edema in the axillary and inguinal regions and often extending over the entire subcutaneous region of the belly, and enlarged hyperemic adrenals. Examination was made as soon after death as possible and cultures from the spleen, liver, and adrenals, and in many cases from the heart blood and kidney, were made to determine the power of penetration, with a view to correlating it with virulence. In taking the cultures, the surface of the organ was cauterized, a small incision made with sterile knife, and some of the pulp transferred to a tube of 0.5% dextrose broth with a reaction of +0.5. These tubes were incubated at 37 C. for 4 or 5 days, and then transfers made to Loeffler's serum. After 24 hours' incubation the serum growth was stained with methylene blue for diphtheria

[44] Roedelius, ibid., 1913, 15, p. 497.
[45] Am. Jour. Dis. Child., 1913, 5, p. 317.
[46] Conradi and Bierast, Deutsch. med. Wchnschr., 1912, 34, p. 1580.
[47] Jour. Infect. Dis., 1915, 16, p. 292.

bacilli. (At first the organ pulp was transferred directly to Loeffler's serum also, but this method was not as satisfactory as the one described and was soon discontinued.)

The detailed results are given in Table 6. In all cases in which 4 c.c. were injected, 0.2 c.c. had proved harmless. It will be seen that of the 23 strains tested, 16 possessed high virulence, 4 low virulence, and 3 were avirulent. B. diphtheriae was isolated 8 times from 7 different animals. It was found 4 times in the spleen, and 2 times each in the liver and the adrenals. Five of the 7 animals received 2 c.c. of a strongly virulent strain, while the other two received a large dose of

TABLE 6

TESTS FOR VIRULENCE AND PENETRATION

| Culture | Dose (c.c.) | Results | Recovery of Diphtheria Bacilli from Tissues | | | | |
|---|---|---|---|---|---|---|---|
| | | | Spleen | Liver | Adrenal | Heart | Kidney |
| 0 | 0.2 | Died in 2 days | — | — | — | — | — |
| 122 | 0.2 | Died in 1½ days | — | — | — | — | — |
| 905 | 0.2 | Died in 2½ days | + | — | — | — | — |
| 668 | 0.2 | Died in 3 days | — | — | — | — | — |
| 461 | 0.2 | Died in 2¾ days | — | — | — | — | — |
| 1290 | 4.0 | Died in ¾ day | + | — | + | | |
| 694 | 4.0 | Lived | | | | | |
| 1303 | 0.2 | Died in 1¼ days | + | — | — | | |
| 1 | 0.2 | Died in 1½ days | — | — | — | | |
| 168 | 0.2 | Died in 1¾ days | — | — | — | | |
| 880 | 0.2 | Died in 2¼ days | — | + | — | | |
| 540 | 4.0 | Died in ¾ day | — | — | + | — | — |
| 42 | 0.2 | Died in 3¾ days | — | + | — | | |
| 600 | 4.0 | Died in 1½ days | — | — | — | — | — |
| 1293 | 0.2 | Died in 1½ days | — | — | — | | |
| 1441 | 0.2 | Died in 1¾ days | — | — | — | | |
| Park's 8 | 4.0 | Died in 2¾ days | — | — | — | | |
| 49 | 4.0 | Lived | | | | | |
| 50 | 4.0 | Lived | | | | | |
| 2059 | 0.2 | Died in 1¼ days | — | — | — | | |
| 11 | 0.2 | Died in 1¼ days | — | — | — | | |
| 13 | 0.2 | Died in 1½ days | — | — | — | | |
| 20 | 0.2 | Died in 2 days | .. | — | — | | |

organisms of rather low virulence. Since the 5 strains the organisms of which were recovered in the tissues showed no higher virulence than the 11 other strains, in which penetration was not found, it would seem that penetration is only a minor factor in the virulence of B. diphtheriae. The postmortem findings were typical of toxemia. It may be that bacteriemia, when present, is only temporary, and that blood examinations at intervals following injection, as suggested by Conradi and Bierast,[46] would give a larger proportion of positive results.

A series of experiments was conducted to determine whether the virulence of an avirulent strain could be increased either by animal passage alone, by symbiosis during passage with Staphylococcus aureus, or by injecting the culture together with sublethal doses of toxin into

successive guinea-pigs. For this purpose Strains 49 and 50 (described by Brown and Smith[48]) were selected, because they were apparently true B. diphtheriae rather than B. hofmanni, it being deemed more probable that virulence would be increased in such strains than in the pseudodiphtheria bacilli. These strains were nontoxic, or nearly so, and avirulent. By Heine's classification, as may be seen by reference to Table 4, they belong to his first group, that should be virulent if the biometric test were taken as a guide, since both ferment dextrose, maltose, dextrin, and glycerin, but produce an alkaline reaction in saccharose. Both strains, when tested for virulence by the method described, produced no general or local effect. They were consequently considered nonvirulent.

In the first series each of the two strains was passed consecutively through 8 guinea-pigs weighing 250-300 gm., a 24-hour Loeffler culture being used for injection in each case. Four standard loops of the culture were added to 1 c.c. of sterile salt solution in a Hitchen syringe and the uniform suspension injected subcutaneously. After 48 hours the site of inoculation was sterilized with alcohol, a small incision made with sterile knife, and a platinum needle inserted, on which cultures were taken for transfer to Loeffler's slants. These were incubated, examined for purity, and another guinea-pig injected with the recovered culture. This series of tests was designed to show any increase in virulence due to passage alone and also to serve as a control for the other passages which were carried on simultaneously with Staphylococcus aureus and toxin. On the 7th passage, Strain 49 changed noticeably, from a long slender organism with few granules to a shorter highly granular organism. It showed no virulence when 4 c.c. of a 48-hour broth culture were injected subcutaneously into a guinea-pig. Strain 50 did not change in appearance with passage and was avirulent when injected in 4-c.c. amounts of a 48-hour broth culture. A change in morphology of these strains had been observed by Brown and Smith at different times during the 12 years the strains were in stock on artificial media, and the change is probably of no significance in this case.

In the second series of experiments the two atoxic strains, in association with Staphylococcus aureus, were passed through 8 guinea-pigs. Four standard loops of the diphtheria culture from a 24-hour Loeffler's slant were added to 1 c.c. of sterile salt solution in a Hitchen syringe with 2 loops of a 24-hour agar-slant culture of Staphylococcus aureus. This was thoroughly mixed and then injected subcutaneously into 250-

[48] Jour. Med. Research, 1914, 30, p. 443.

300-gm. guinea-pigs. After 48 hours a small incision was made asepti-
cally at the site of inoculation and some of the edematous material
taken with a looped needle and spread on Loeffler slants. After 24-48
hours the diphtheria colonies could usually be distinguished in some of
the tubes and transferred to a sterile tube. This culture, after it had
been examined for purity, was used for the next passage. The staph-
ylococcus culture was always taken from the original culture and not
from one passed through the series of guinea-pigs. By this method
Strain 49 changed at the 5th passage, to a highly granular shorter form.
It was not virulent when tested after the 8th passage in 4-c.c. amounts
of a 48-hour broth culture. Strain 50 with Staphylococcus aureus did

TABLE 7

THE EFFECT OF ANIMAL PASSAGE AND SYMBIOSIS ON VIRULENCE

| Treatment | Changed Morphology During Treatment | Virulence |
|---|---|---|
| **Strain 49** | | |
| Before passage.................................. | .......................... | |
| 8 passages alone.............................. | After 7th passage | |
| 8 passages with Staphylococcus aureus......... | After 5th passage | Avirulent in 4-c.c. amounts |
| 8 passages with ½ M. L. D. toxin.............. | .......... ......... | |
| **Strain 50** | | |
| Before passage................... .. ........ | ...................... | |
| 8 passages alone................................. | ...................... | Avirulent in 4-c.c. amounts |
| 8 passages with Staphylococcus aureus......... | ...................... | Local abscess containing pure growth of diphtheria |
| 7 passages with ½ M. L. D. toxin........ ..... | ...................... | bacilli. No toxemia |

not change in morphology during passage. After the 8th passage the
strain had acquired pyogenic properties; when 4 c.c. of a 48-hour broth
culture were injected subcutaneously a nodule, the size of a hazelnut,
formed at the site of injection. From this nodule, which contained
purulent material, the diphtheria bacilli were recovered in pure growth
after 4 days. No general symptoms of toxemia developed. This action
is probably similar to that observed by Cobbett,[39] and Councilman,[40]
and suggested by Heine as a property of his second group; it may
indicate a low grade of virulence.

In the third series the two strains were passed through a series of
guinea-pigs together with ½ M.L.D. of diphtheria toxin. Strain 49
was passed through 8 guinea-pigs and Strain 50 through 7. Four loops

of a 24-hour Loeffler growth were injected subcutaneously, each time with ½ M.L.D. of sterile toxin, and the diphtheria bacilli recovered after 48 hours from the site of injection. Strain 49 did not change morphologically during passage and was avirulent after 8 passages. Strain 50 became more granular and shorter after the 5th passage, and produced, after the 7th passage, a local abscess at the site of injection from which a pure growth of diphtheria bacilli was isolated. No general toxic effects were observed.

The results of these experiments, under the conditions established, may be summed up as follows: (1) passage through susceptible animals did not increase virulence even in strains whose biometric reactions are typically those of true diphtheria; (2) passage with Staphylococcus aureus may have caused the acquisition of a low grade of virulence in some cultures; (3) passage with toxin may also have caused the acquisition of a low grade of virulence in some cases.

It was thought worth while to determine the number of organisms that are needed to produce fatal results within 4 days, and to demonstrate the relation that this might bear to virulence. The difficulties encountered in accurately determining the number of bacilli injected have been manifold. Four methods have been tested for counting the organisms injected:

(1) The standard platinum-loop method. It was very soon found that the dry growth of diphtheria bacilli on Loeffler's serum could be measured with no degree of accuracy with the platinum loop.

(2) Wright's method for standardizing vaccines was employed—washing down the growth on a Loeffler's tube, breaking up the clumps with sterile glass beads in a test tube, and then counting the organisms stained with methylene blue diluted 5 times and comparing the result with the number of erythrocytes. In all cases 500 organisms were counted from two slides. This method gives reasonable satisfaction, tho there are certain objections to its use; living bacteria cannot be distinguished from dead, and there is often great difficulty in obtaining a uniform distribution of blood and organisms, resulting in an unintentional selection of fields to be counted in an effort to find thin fields.

(3) One-percent-glycerin agar plates were made of the diluted suspension and the bacteria counted after 48 hours' incubation at 37 C. This method has the advantage of showing only living organisms, but it may be questioned whether all living organisms will grow sufficiently in glycerin agar to be counted at 48 hours, for, as is well known, diphtheria bacilli do not grow vigorously on agar and strains differ in their ability to grow on this medium.

(4) The organisms were washed down in sterile salt solution, shaken thoroughly with glass beads, and the organisms counted with a Thoma hemacytometer. Methylene blue diluted 5 times was used as a diluent and stain, and the organisms were counted with a Leitz No. 7 objective and No. 4 ocular. This method is open to the same objection as Wright's method in not distinguishing between living and dead organisms. Altogether, however, it

was considered the most satisfactory of the methods employed. Any method of counting diphtheria organisms is rendered difficult by the well-known tendency of the organisms to clump. This difficulty was eliminated as much as possible by shaking thoroughly with glass beads. The objection that the 2nd and 4th methods do not distinguish between living and dead organisms has been met by using only 24-hour cultures, in which a maximal number of organisms are viable. It is not contended that for any culture tested the three methods give identical results, because it has been found that there may be high percentages of difference.

The minimal fatal doses of living organisms of 5 different strains were carefully determined by injecting subcutaneously into standard guinea-pigs known numbers of organisms, obtained by the plating and blood-counter methods. These results are compared in Table 8 with

TABLE 8

RELATION OF THE NUMBER OF ORGANISMS INJECTED TO VIRULECE

| Culture | Virulence (shown by injection of 48-hour broth culture) | Organisms Necessary for Fatal Results | | | |
|---|---|---|---|---|---|
| | | Fraction of 24-hour Loeffler Slant | Number by Blood Counter | Fraction of 24-hour Loeffler Slant | Number by Plating |
| Park's  8 | Low | 1.0 | 1,930,000,000 | 2.4 | 152,000,000* |
| 2059 | High | 0.0004 | 6,592,000 | 0.01 | 19,900,000 |
| 11 | High | 0.0001 | 1,216,000 | 0.001 | 1,210,000 |
| 13 | High | 0.001 | 1,520,000 | 0.001 | 780,000 |
| 20 | High | 0.02 | 160,000,000 | 0.005 | 15,650,000 |

* Not fatal.

the results for the same strains determined by the routine method described earlier in this paper. It will be seen that the number of organisms necessary to produce fatal results was much greater for a strain of low virulence, such as Park's No. 8, than for the other four highly virulent strains. In one case the growth on 2.4 Loeffler slants washed down in salt solution did not produce fatal results, but subsequently the same strain from another laboratory produced fatal results when the growth from one tube was injected. A 4-c.c. dose of a 48-hour broth culture of this organism was uniformly fatal. Inasmuch as such variations do occur when washed cultures are injected, it would seem advisable to adopt uniform methods of testing for virulence in public health laboratories. Undoubtedly, the virulence in this case when tested with the broth culture was due to the toxin contained in the broth. This culture, however, might have been classed as avirulent had the washed-down growth alone been used.

Experiments were performed after the methods of Rosenow[10] to ascertain whether there were virulins present in the highly virulent

strains that could increase the virulence of low-virulent strains or render sublethal doses of virulent organisms lethal.

Strain 11 was selected for the production of virulins because of its high virulence. The organisms were grown on the surface of 15 Loeffler tubes for 24 hours, and then were washed down in a total volume of 50 c.c. $\frac{M}{8}$ NaCl. This emulsion was kept in the incubator for 48 hours at 37 C. and then was filtered through a Berkefeld filter and tested for sterility. This constituted Virulin A. Virulin B was prepared by washing down 15, 24-hour Loeffler tubes with 50 c.c. of sterile water. This was kept in the incubator for 6 hours and then rapidly frozen and thawed 4 times. The suspension was then centrifugated and the clear supernatant fluid filtered through a Berkefeld filter. The strains used with the virulins were Park's No. 8, and Strains 13, 11, and 20. These were grown on Loeffler slants and an emulsion made in sterile salt solution. Sublethal doses, or doses approximating the lethal, were injected together with the virulin.

Table 9 shows that no increase in virulence was induced in any case except in Strain 20 with Virulin A. The guinea-pig in this case died on the 10th day, while the control animal lived. In 2 cases the virulin seemed to have some protective action. In general, it may be said that the virulin did not influence the virulence to any degree.

The Bail method of obtaining aggressins was also employed.

TABLE  9

'VIRULINS' AND THEIR EFFECT ON VIRULENCE

| Strain | Proportion of Loeffler Slant in 1 c.c. NaCl | Amount of Virulin (c.c.) | Results |
|---|---|---|---|
| Control | 0 | 3 Virulin A | Lived. No local action |
| Park's 8 | 0.2 | 3 Virulin A | Lived. Marked necrosis and edema |
| Park's 8 | 0.2 | 0 (3 NaCl) | Lived. Marked necrosis and edema |
| 13 | 0.0001 | 3 Virulin A | No local action |
| 13 | 0.0001 | 0 (3 NaCl) | No local action |
| 11 | 0.0001 | 3 Virulin A | Died in 10 days. Severe edema |
| 11 | 0.0001 | 0 (3 NaCl) | Died in 4 days. Edema and necrosis |
| 20 | 0.0002 | 3 Virulin A | Died in 10 days. Edema and necrosis |
| 20 | 0.0002 | 0 (3 NaCl) | Lived. Edema and necrosis |
| Control | 0.00 | 3 Virulin B | No local action |
| Park's 8 | 0.2 | 3 Virulin B | Lived. Edema and necrosis |
| Park's 8 | 0.2 | 0 (3 NaCl) | Lived. Edema and necrosis |
| 13 | 0.0001 | 3 Virulin B | Lived. Edema and necrosis |
| 13 | 0.0001 | 0 (3 NaCl) | Died in 3 days. Animal accidentally destroyed without autopsy |

Rabbits were first used in hopes of obtaining a large amount of peritoneal exudate. One cubic centimeter of a 48-hour culture on sugar-free broth of a highly virulent strain, No. 2059, was injected intraperitoneally into each of 2 rabbits and the same amount of another virulent strain, No. 11, was injected into each of 2 other rabbits. These rabbits were bled to death after 4 days and the peritoneal exudate taken. Very little exudate had been formed, altho there was a membrane present on the viscera of all the rabbits. A total of 5 c.c. was collected. The blood was defibrinated as drawn and pooled from the

4 rabbits for the tests. The corpuscles were thrown down by centrifugation, as well as the cellular material in the peritoneal exudate. The organisms in the exudate were killed by 5% phenol, the fluid was tested for sterility by plating, and tests were made with the exudates and serum as outlined in Table 10. With the small amount of material it was impossible to make tests

TABLE 10

'AGGRESSINS' AND THEIR INFLUENCE ON B. DIPHTHERIAE

| Culture | Proportion of Loeffler Slant in 1 c.c. Fluid | Amount of Aggressins (c.c.) | Amount of NaCl Solution (c.c.) | Result |
|---|---|---|---|---|
| 49 (passed with Staphylococcus aureus through 8 guinea-pigs) | 0.33 | 3 (rabbit blood serum) | 0 | |
| Serum control............. | 0 | 3 (rabbit blood serum) | 1 | |
| 49 (passed with Staphylococcus aureus through 8 guinea-pigs) control | 0.33 | 0 | 3 | |
| 49 (passed with Staphylococcus aureus through 8 guinea-pigs) | 0.33 | 2 (rabbit peritoneal fluid) | 1 | No effect |
| R a b b i t-peritoneal-fluid control | 0 | 2 (rabbit peritoneal fluid) | 2 | |
| 50 (passed with toxin through 6 guinea-pigs) | 0.33 | 3 (guinea-pig peritoneal fluid) | 0 | |
| 50 (passed with toxin through 6 guinea-pigs) control | 0.33 | 0 | 3 | Lived |
| Guinea-pig-peritoneal-fluid control | 0 | 3 (guinea-pig peritoneal fluid) | 1 | |
| Park's No 8................ | 0.2 | 3 (guinea-pig peritoneal fluid) | 0 | Slight edema |
| Park's No. 8 control........ | 0.2 | 0 | 3 | |
| 49 (passed with Staphylococcus aureus through 8 guinea-pigs) | 0.33 | 3 (guinea-pig peritoneal fluid) | 0 | No effect |
| 49 (passed with Staphylococcus aureus through 8 guinea-pigs) control | 0.33 | 0 | 3 | |

with controls on more than 1 strain. Strain 49, which in conjunction with Staphylococcus aureus had been passed through 8 guinea-pigs, was used. This strain had changed in morphology during passage, but not in virulence. It will be seen that the exudate caused no increased virulence in this case.

Since the peritoneal exudate in rabbits was so slight, guinea-pigs were substituted. Six large guinea-pigs were injected intraperitoneally with the same strains, Nos. 2059 and 11, 1 c.c. of a 48-hour broth culture being used in each

case. One animal injected with each strain died before the expiration of 4 days; the rest were killed at that time and the peritoneal exudate taken. The amount of exudate varied in the different guinea-pigs from 2 to 15 c.c., but all showed some lesions and membrane. After all the fluid possible had been obtained, the viscera and peritoneal cavity of each guinea-pig were washed with 10 c.c. of salt solution and the washing added to the exudate. The combined fluid was centrifugated and the supernatant fluid sterilized by passing through a Berkefeld filter. Three strains were used to test the potency of the filtrate—Strain 50 after 6 passages through guinea-pigs with toxin, Park's No. 8, and Strain 49, which together with the staphylococcus had been passed through 8 guinea-pigs.

The results are recorded in Table 10. In no case did the aggressin increase the virulence.

### THE RELATION BETWEEN BIOMETRIC REACTIONS AND VIRULENCE

The fermentation reactions of diphtheria bacilli have been considered one of the most satisfactory bases of classification within the diphtheria group. There is little question but that true diphtheria bacilli ferment dextrose much more vigorously than do the pseudo-diphtheria bacilli, and that strong dextrose-fermenters are more likely

TABLE 11

RELATION BETWEEN BIOMETRIC REACTION AND VIRULENCE

| Carbohydrate | Reaction | Virulence | | | | |
|---|---|---|---|---|---|---|
| | | High | Medium | Low | Avirulent | Not Tested |
| Dextrose.......... | 2 below 3.5.......... | 1 | 0 | 0 | 0 | 1 |
| | 19 from 3.5 to 4.5.... | 12 | 1 | 2 | 2 | 2 |
| | 5 over 4.5............ | 3 | 0 | 1 | 1 | 0 |
| | 15 acid.............. | 8 | 1 | 2 | 3 | 1 |
| Maltose........... | 2 neutral........... | 2 | 0 | 0 | 0 | 0 |
| | 9 alkaline........... | 6 | 0 | 1 | 0 | 2 |
| | 3 acid.............. | 2 | 1 | 0 | 0 | 0 |
| Saccharose........ | 2 neutral........... | 1 | 0 | 0 | 1 | 0 |
| | 21 alkaline........... | 13 | 0 | 3 | 2 | 3 |
| | 10 acid.............. | 8 | 0 | 0 | 2 | 0 |
| Dextrin........... | 3 neutral........... | 2 | 0 | 0 | 0 | 1 |
| | 13 alkaline........... | 6 | 1 | 3 | 1 | 2 |
| | 20, 2 or below........ | 13 | 1 | 2 | 1 | 3 |
| Glycerin........... | 6 over 2............. | 3 | 0 | 1 | 2 | 0 |

to be virulent. No test other than a virulence test has as yet been found by which true virulent diphtheria bacilli can be differentiated from the diphtheria bacilli that are avirulent. Heine[17] has sought to distinguish such cultures by their fermentation results on different sugars. His 1st group of virulent organisms ferments dextrose,

maltose, lactose, and dextrin, but not saccharose.  Lactose, which forms
one of his important differential sugars, was not used with the 26
strains that were tested in my experiments.  That being left out of
consideration, 10 of the 26 strains belonged in this group; 7 of these
were highly virulent, 2 were avirulent, and 1 was not tested.  Three of
the other strains probably belonged to Heine's 2nd and 3rd groups; of
these, 2 were highly virulent and 1 was of medium virulence.  A sum-
mary of the relation between virulence and fermentation on 5 carbo-
hydrates is given in Table 11.  From this table it will be seen that there
is no constant relation between these two characteristics in dextrose,
maltose, saccharose, dextrin, and glycerin.  The greater number of
highly virulent strains produce between 3.5 and 4.5% of normal acid
in dextrose, give acidity in maltose and dextrin, and less than 2% acid
in glycerin, with alkalinity in saccharose.  In none of these carbohy-
drates is there a constant production of definite acidity or alkalinity
among highly virulent strains.  None of the strains that were of low
virulence or avirulent, however, produced any acid in saccharose, altho
2 virulent strains fermented this sugar.

### TOXIN-PRODUCTION IN RELATION TO VIRULENCE

The factors that enter into the production of toxin are as complex
and varied as those that enter into virulence.  Indeed, many writers
have made no distinction between toxicity and virulence, using the
terms synonymously.  Nuttall and Graham Smith[18] state: "It cannot
be said that there is any very marked correspondence between the
severity of the disease in the human subject and the virulence or
toxigenic power of the bacillus obtained from the case."  Moshage and
Kolmer[1] believe that acid-production may run parallel to virulence.
Neisser and Ginns[49] state that often diphtheria bacilli that are
extremely toxic to the guinea-pig may be rather mildly virulent for
man.  Sometimes also cultures virulent for man are only mildly toxic
to the guinea-pig.  Such statements naturally bring up the question as
to what relation may exist between toxicity and virulence.

It is conceivable that virulent strains which produce a feeble toxin
in vitro may be able to produce a strong toxin in vivo that may be the
sole factor in virulence, for it is well known that slight changes in the
broth composition profoundly affect the amount of toxin produced.
It does seem, however, that these factors whatever they might be,

[49] Kolle and Wassermann, Handb. d. pathogen. Microorganismen, 1913, 5, p. 962.

would be operative alike in different strains; for example, two equally virulent strains would be expected to produce toxin in vitro in the same broth under identical conditions in about the same degree, if toxin alone were responsible for their virulence. Some have thought that in certain strains the toxin may be contained largely in the bodies of the bacilli. It has been reported, however, that the bodies of the diphtheria bacilli do not at any time contain toxin in any considerable amounts.[50] This is also indicated by my virulin experiments, in which the extracted fluid seemed to be entirely without toxic effects.

The demand for diphtheria antitoxin has resulted in much fruitful work in the production of toxins. Hida[51] called attention to the importance of the kind of peptone used, having found that a peptone containing deutero-albumose in considerable amounts gives the most potent toxins. Park and Williams[50] found, as have others since, that 2% of peptone gives the best results. The importance of good peptone in toxin-production has been further emphasized since the supply of Witte peptone was cut off by the war. I found, as did others, that the ability of a strain to produce a potent toxin was considerably lower when certain American-made peptones were used in the medium than when the Witte peptone was employed. The product of the autodigestion of hog stomach has given very good results when used as a substitute for peptone. The kind of meat used for the infusion broth has in many cases had an effect on the strength of the toxin. Usually beef infusion or veal broth is used, many preferring the latter for obtaining a high toxin. It has been found that proteins were not essential to toxin-formation, that a potent toxin could be formed on nonprotein synthetic media, one which would kill the guinea-pig in 36-38 hours.[52] Other conditions, as the amount of dextrose, have been emphasized by Spronck[53] as of prime importance in toxin-production. The relation of oxygen and the production of a heavy surface growth have been discussed by different writers. Undoubtedly all of these factors do play a part in the production of toxin. The production of a heavy surface film does not necessarily indicate a strong toxin, altho a strong toxin is rarely formed unless a good surface growth occurs. Park and Williams[50] state that in their experiments, when other conditions were similar, the strength of the toxin was in proportion to the virulence and vigor of the growth of bacilli employed.

Strains of diphtheria bacilli isolated from clinical cases vary widely in their ability to produce toxin. It is not difficult to get toxins with an M. L. D. of 0.1, 0.05, or even 0.03 c.c. from such sources, but strains producing a toxin with an M. L. D. of less than 0.01 c.c. are very rare. The Park strain has, however, continued to give a very strong toxin, 0.007-0.001 for two decades. Fruitless efforts have been made by Smith and Walker[54] and Berry and Blackburn[55] to find a strain producing a stronger toxin than this. Of the 46 cultures tested by Smith and Walker, 12 gave an M. L. D. between 0.036 and 0.06, 21 between

[50] Park and Williams, Jour. Exper. Med., 1896, 1, p. 164.
[51] Ztschr. f. Hyg. u. Infektionskr., 1908, 61, p. 273.
[52] Hadley, Jour. Infect. Dis., 1907, Suppl. 3, p. 95.   Uschinsky, Centralbl. f. Bakteriol., 1897, 21, p. 146.
[53] Ann. de l'Inst. Pasteur, 1895, 9, p. 758.
[54] Jour. Med. Research, 1897-98, 2, p. 12.
[55] Jour. Infect. Dis., 1912, 10, p. 404.

0.07 and 0.09, and 9 between 0.10 and 0.12. Of the 100 cultures tested by Berry and Blackburn, 9 did not produce fatal results when 3 c.c. were injected, 27 had an M. L. D. of 1 c.c., 11 of 0.05, 6 of 0.033, 25 of 0.02, 11 of 0.01, and 3 of 0.005. It is pointed out that the Park strain grown under the same conditions has varied in the toxin produced from an M. L. D. of 0.05, to 0.003, 0.0015, and 0.0005 c.c.[56] Indeed, it is rare to find 2 flasks growing under identical conditions that give the same M. L. D., altho the variation may be slight.

In order to correlate virulence, toxin-production, and acid-production, toxin tests were made on a number of different strains some of which were avirulent, some of low virulence, and some of high virulence. The production of toxin at the time this study was undertaken was complicated by the use of an American peptone which was of little value in producing toxin. Park's strain, and Strains 20, 11, and 2059 did not produce enough toxin with this peptone to kill a guinea-pig in repeated tests with 1-c.c. amounts, while of Strain 11, 0.7 c.c. was required to produce fatal results. Later, when Witte peptone was substituted, strong toxins were obtained from these same strains.

The methods of producing toxins were as follows: The medium used was beef infusion broth prepared in the usual way, and rendered sugar-free. This was titrated carefully to + 1.2, and sterilized by the discontinuous method in 250-c.c. Erlenmeyer flasks containing 75 c.c. of the medium. Sufficient 10% sterile dextrose solution was then added to each flask to make a dextrose content of 0.2%. Some of the same medium was filled into test tubes and the organisms trained to grow on the surface before transference to the flasks. After the surface growth was transferred to the flasks, the latter were incubated at 35-36 C. for 10 days. The flasks were then examined for purity of growth and if pure, 0.5% phenol was added and the flasks placed in a dark ice box over night. The material was filtered the next morning through filter paper and then through a Chamberland filter, and tested for purity. Initial tests were made by injecting subcutaneously 1, 0.7, 0.3, and 0.1 c.c. of the toxin into 250-300-gm. guinea-pigs. All animals that died were examined and are included only if there was typical toxemia.

Table 12 gives the results of these tests, together with the virulence and biometric results for the same strains. The strongly virulent strains all produced strong toxins, strains with low virulence in all cases produced fatal toxins, while avirulent strains in all cases failed to produce any toxins that were lethal in large doses. One low-virulence strain, Park's No. 8, produced the most potent toxin, while the other three of low virulence were not such strong toxin-producers as three of the high-virulence strains. Of the toxin-producers, all fermented dextrose and glycerin, 7 fermented maltose, 2 saccharose,

[56] Kolle and Wassermann, Handb. d. pathogen. Microorganismen, 1913, 5, p. 965.

and 5 dextrin; of the non-toxin-producers, all produced acid in dex-
trose, maltose, dextrin, and glycerin, and none fermented saccharose.
The number of strains tested is too few to permit of any far-reaching
conclusions, but it does not seem that the acid-production in the five
sugars can be taken as an index of toxin-production.

TABLE · 12

TOXIN-PRODUCTION AND VIRULENCE

| Culture | Biometric Reactions | | | | | Virulence | Toxin M. L. D. |
|---|---|---|---|---|---|---|---|
| | Dextrose | Maltose | Saccharose | Dextrin | Glycerin | | |
| 20 | + | + | — | + | + | High | 0.05 |
| 13 | + | + | — | + | + | High | 0.1 |
| 11 | + | + | + | + | + | High | 0.0075 |
| 2059 | + | + | — | + | + | High | 0.5 |
| Park's 8 | + | + | — | + | + | Low | 0.0075 |
| 540 | + | — | — | — | + | Low | 0.7 |
| 600 | + | + | — | — | + | Low | 0.3 |
| 1290 | + | + | + | — | + | Low | 0.3 |
| 694 | + | + | 0 | — | + | Avirulent* | 1.0 No action |
| 50 (passed 6 times with toxin) | + | + | — | + | + | Very low.* Abscess-formation | 4.0 No action |

* Not fatal.    + = acid; — = alkaline.

The relationship between the number of organisms that are neces-
sary to produce fatal results and the toxin-production is seen in Table
13.   Five strains only are compared.   The virulence and the number
of organisms necessary to produce fatal results run in nearly reverse
order.   There is apparently no relationship between the minimal fatal

TABLE   13

RELATIONSHIP  BETWEEN  THE  NUMBER  OF  ORGANISMS  INJECTED,  TOXIN-PRODUCTION,  AND
VIRULENCE

| Strain | Number of Organisms Necessary for Death | Toxin M. L. D. | Virulence 48-hour Broth Culture |
|---|---|---|---|
| Park's No. 8 | 1,930,000,000 | 0.0075 | Low |
| 20 | 160,000,000 | 0.025 | High |
| 13 | 1,520,000 | 0.05 | High |
| 11 | 1,216,000 | 0.0075 | High |
| 2059 | 6,592,000 | 0.5 | High |

dose of live organisms and the M.L.D. of toxin, for the Park strain is
the strongest toxin-producer, yet a larger number of organisms are
required to produce fatal results.   Strain 2059 has relatively weak
toxigenic powers, yet fewer bacteria are necessary for fatal results than
in the case of Strain 20, which has double the toxigenic powers.

SUMMARY AND CONCLUSIONS

There is a maximal acid-production for each strain in various carbohydrates. The greater the relative exposure to oxygen of the air, the more rapid is the production of acid.

The time of the maximal production of acid in the various carbohydrates varies not only with the carbohydrate used, but also with different strains. A uniform time, 8 days, for reading the final results in all carbohydrates will give the characteristic biometric results. The lack of uniformity in the reported results of various workers may be due to the use of different indicators and to reading the results at different periods of incubation.

A dextrose, maltose, saccharose, or dextrin content of 0.5% gives as satisfactory biometric results as of 1%.

The biometric results on 26 strains of morphologically true diphtheria show that all fermented dextrose and glycerin, the majority (23) produced no acid in saccharose, 15 fermented maltose, and 13 dextrin.

Twenty-three strains were tested for virulence and the power of penetration in order to determine whether penetration and virulence are related. Sixteen strains were highly virulent, 4 possessed low virulence, and 3 were avirulent. Bacilli were recovered from the tissues 8 times. The virulence of the culture seemed to be of less importance in relation to penetration than the number of organisms injected.

No increase in virulence was produced in two morphologically and biometrically typical atoxic strains by passage through 8 successive guinea-pigs.

When the same avirulent strains were passed, together with Staphylococcus aureus, through 8 successive guinea-pigs, one of the two acquired slight pyogenic powers for the guinea-pig, but produced no general effect or toxemia. The other strain was unchanged.

Passage of the same avirulent strains together with ½ M.L.D. of toxin through 7 and 8 successive guinea-pigs produced pyogenic powers in the same strain that was modified by passage with staphylococcus. This may indicate a very low grade of virulence.

The use of the Thoma hemacytometer gives the most satisfactory results in counting the number of diphtheria bacilli injected. The bacilli are well stained for counting in Loeffler's methylene blue diluted 5 times.

The minimal fatal dose of living diphtheria bacilli seems to be greater in strains of low virulence.

Some strains of bacilli may be classed as avirulent when tested by using the washed-down growth from a Loeffler tube that would be considered virulent if tested by injection of a 48-hour broth. This difference is undoubtedly due to toxin formed in the broth.

'Virulins' prepared by a method similar to that employed by Rosenow did not modify the virulence when injected with sublethal doses of virulent organisms.

In no case did 'aggressins' prepared by the Bail method increase the virulence or produce fatal results when injected with sublethal doses of virulent strains.

No constant relationship exists between virulence and the fermentation reactions in dextrose, maltose, saccharose, dextrin, and glycerin. All virulent strains (and many avirulent) ferment dextrose and glycerin, usually give an acid reaction in maltose and dextrin, but do not ferment saccharose. Exceptions occur, however, in the fermentative action of virulent strains on maltose, dextrin, and saccharose.

The biometric reactions cannot be taken as indicative of toxin-production. They do not run parallel.

Toxin-production and virulence do not run parallel in many cases. The number of organisms necessary for fatal results is a good indication of the virulence, but gives no idea of the toxicity of the strain.